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PTO/SB/05 (08/00)
Approved for use through 10/31/2002, OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket	920920.90045
First Inventor	Chawnshang Chang
Title	Mutual Suppression Between Sex Hormone Receptors ...
Express Mail Label No.	EK941817725US

APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents.

ADDRESS TO: **Commissioner for Patents, Box Patent Application, Washington, D.C. 20231**

1. [X] Fee transmittal Form (Submit an original and a duplicate for fee processing)
2. [ ] Applicant claims small entity status. See 37 CFR 1.27.
3. [X] Specification [Total Pages 27]
   (preferred arrangement set forth below)
   - Descriptive title of the invention
   - Cross References to Related Applications
   - Statement Regarding Fed Sponsored R&D
   - Reference to sequence listing, a table or a computer program listing appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
4. [X] Drawing(s) (35 USC 113) [Total Sheets 10]
5. Oath or Declaration [Total Pages 2]
   a. [X] Newly unexecuted (original or copy)
   b. [ ] Copy from prior Application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed)
      i. [ ] DELETION OF INVENTOR(S) Signed Statement attached deleting inventor(s) named in prior application, see 37CFR 1.63(d)(2) and 1.33(b).
6. [ ] Application Data Sheet. See 37 CFR 1.76
7. [ ] CD-ROM or CD-R in duplicate, large table or Computer Program (Appendix)
8. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
   a. [ ] Computer Readable Form (CRF)
   b. *Specification Sequence Listing on*
      i. [ ] CD-ROM or CD-R (2 Copies); or
      ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

9. [ ] Assignment Papers (cover sheet & documents)
10. [ ] 37 CFR 3.73(b) Statement (where there is an assignee) [ ] Power of Attorney
11. [ ] English Translation Document *(if applicable)*
12. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
13. [ ] Preliminary Amendment
14. [X] Return receipt postcard (MPEP 503) *(Should be specifically itemized)*
15. [ ] Certified copy of priority Document(s) *(if foreign priority is claimed)*
16. [ ] Other:

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information: and in a preliminary amendment or in an Application Data Sheet under 37 CFR 1.76

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application no. ___/_____

Prior application information: Examiner: Group/Art Unit:

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

18. CORRESPONDENCE ADDRESS

[ ] Customer Number or Bar Code Label (Insert Customer No. or Attach bar code label here) or [X] Correspondence address below

NAME	Nicholas J. Seay				
	Quarles & Brady LLP				
ADDRESS	1 South Pinckney Street				
	P O Box 2113				
CITY	Madison	STATE	WI	ZIP CODE	53701-2113
COUNTRY	USA	TELEPHONE	608/251-5000	FAX	608/251-9166

Name	Nicholas J. Seay	Registration No. (Attorney/Agent)	27,386
Signature		Date:	November 13, 2000

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

FEE TRANSMITTAL for FY 2000

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT $ 1,190.00

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	Chawnshang Chang
Group Art Unit	
Examiner Name	
Attorney Docket Number	920920.90045

METHOD OF PAYMENT (check one)

1. [X] The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:

Deposit Account Number: 17-0055

Deposit Account Name: Quarles & Brady LLP

[X] Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17

[ ] Applicant claims small entity status See 37 CFR 1.27

2. [ ] Payment Enclosed:

[ ] Check [ ] Credit Card [ ] Money Order [ ] Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	710	201	355	Utility filing fee	710.00
106	320	206	160	Design filing fee	
107	490	207	245	Plant filing fee	
108	710	208	355	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) ($)710.00

2. CLAIMS

			Extra		Fee from below		Fee Paid
Total Claims	15	-20**=	0	X		=	
Independent	9	-3**=	6	X	80	=	480
Multiple Dependent Claims						=	

** or number previously paid, if greater, For reissues see below

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	80	202	40	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim
109	80	209	40	**Reissue independent claims over original patent
110	18	210	9	**Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)480.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920	112	920	Requesting publication of SIR prior to Examiner action	
113	1,840	113	1,840	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	390	216	195	Extension for reply within second month	
117	890	217	445	Extension for reply within third month	
118	1,390	218	695	Extension for reply within fourth month	
128	1,890	228	945	Extension for reply within fifth month	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive unavoidably abandoned application	
141	1,240	241	620	Petition to revive unintentionally abandoned application	
142	1,240	242	620	Utility issue fee (or reissue)	
143	440	243	220	Design issue fee	
144	600	244	300	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	710	246	355	Filing a submission after final rejection (37 CFR 1.129(a))	
146	710	246	355	For each additional invention to be examined (37 CFR 1.129(b))	
179	710	270	355	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	
Other fee (specify)					

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($).00

SUBMITTED BY				Complete (if applicable)	
Typed or Printed Name	Nicholas J. Seay	Registration No. (Attorney/Agent)	27,386	Telephone	608/251-5000
Signature				Date	November 13, 2000

MUTUAL SUPPRESSION BETWEEN SEX HORMONE RECEPTORS AND OTHER NUCLEAR RECEPTORS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of provisional application Serial No. 60/165,300, filed Nov. 12, 1999.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

To be determined.

BACKGROUND OF THE INVENTION

[0001] The present invention relates the heterodimer formation of nuclear receptors through which the transactivation activity of both receptors of the heterodimer is repressed. In addition, the present invention relates to potential new treatment options and new therapeutic agent screening strategies for nuclear receptor-related diseases or clinical conditions.

[0002] Nuclear receptors are the largest superfamily of eukaryotic transcription factors and include more than 150 proteins so far identified. The superfamily includes intracellular receptors for steroid hormones, thyroid hormones, and retinoids, as well as a large number of orphan receptors for which regulatory ligands have not yet been identified. Regardless of whether transcriptional activity is controlled by the binding of a ligand or not, each of these proteins must be capable of binding to a specific DNA sequence that identifies particular genes as targets for regulation. The interactions between the protein and the DNA are mediated by highly conserved DNA binding domains (DBD) of each protein, the presence of the DBD defining the nuclear receptor superfamily. Interactions between proteins, necessary for the formation of homodimers and/or heterodimers, are mediated by an extensive carboxyl terminal dimerization interface that is contained within the ligand binding domain (LBD) in each of these proteins. Mangelsdorf, et al., *Cell* 83, 851-857 (1995). The members of this superfamily are often classified by the method of dimerization as well as the agent which activates the receptor. Homodimeric receptors in this family include the receptors for androgen (AR), glucocorticoid, estrogen (ER), and mineralocorticoid and a large diverse subfamily of non-steroid receptors including receptors for thyroid hormone, retinoids and vitamin D, as well as many orphan receptors, for which the majority will heterodimerize with retinoid X receptor (RXR). These

RXR heterodimers function as dynamic transcription factors in which one subunit influences the capacity of the other subunit to interact with the ligand and with other co-factors. Forman, et al., *Cell* 81, 541-550 (1995); Kurokawa, et al., *Nature* 371, 528-531 (1994); Schulman et al., *Genes Dev.* 11, 299-308 (1997); Zamir, et al., *Genes Dcv.* 11, 835-846 (1997); Wiebel, & Gustafsson, *Mol. Cell. Biol.* 17, 3977-3986 (1997). Another common heterodimer partner, known as the short heterodimer partner, like RXR, can interact with various nuclear receptors and acts as a negative regulator for the nuclear receptor signaling pathway. Seol, et al., *Science* 272, 1336-1339 (1996).

[0003] The androgen receptor (AR)is a ligand inducible transcription regulator that can activate or repress its target genes by binding to its hormone response elements (HRE) as a homodimer. The AR consists of four major functional domains including a ligand binding domain (LBD), and two activation functions (AF) residing in the N-terminal (AF-1) and the C-terminal end of the LBD (AF-2) respectively.

[0004] Many human diseases and clinical conditions are related to cellular functions mediated by nuclear receptors such as, among others, AR, ER, TR4 and TR2. For example, AR and ER are involved in prostate cancer and breast cancer, respectively. TR4 orphan receptor and TR2 orphan receptor may be involved in neuro-brain function loss through their regulation of the ciliary neurotrophic factor receptor gene. Young, et al., *J. Biol. Chem.* 272, 3109-3116 (1997); Young, et al., *J. Biol. Chem.* 273, 20877-20885 (1998). Other evidence that TR4 and TR2 may be involved in a variety of human clinical conditions come from the fact that they suppress retinoic acid (RA)-induced transactivation, Lin, et al., *J. Biol. Chem.* 270, 30121-30128 (1995), Lee, et al., *J. Biol. Chem.* 273, 13437-13443 (1998), recognize a DNA promoter in Simian virus 40, Lee, & Chang, *J. Biol. Chem.* 2705, 5434-5440 (1995), Lee, et al., *J. Biol. Chem.* 270, 30129-3013 (1995), modulate thyroid hormone and vitamin D signal cascades, Lee, et al., *J. Biol. Chem.* 272, 12215-12220 (1997), Lee, et al., *J. Biol. Chem.* 274, 13437-13443 (1998), and exert negative activities on the erythropoietin gene expression. Lee, & Chang, *J. Biol. Chem.* 271, 10405-10412 (1996). TR4 is also observed to enhance the expression of human hepatitis B virus. Breidbart, et al., *Pediatric Res.* 34, 300-302 (1993). TR2 is observed in most of the developing neural structures, Young, et al., *J. Biol. Chem.* 273, 20877-20885 (1998), suggesting that TR2 plays an important role in the development process of nervous system. In addition, the TR2 mRNA levels are abundantly distributed in many tissues of male rat reproductive organs and highly expressed in mouse embryos beginning at embryonic day 9 and in adult testis, indicating

that the TR2 has a deep involvement in early reproductive functions. Lin, et al., *J. Biol. Chem.* 270, 30121-30128 (1995).

[0005] Modulating nuclear receptor transactivating activity has been proved successful in treating diseases that are related to such nuclear transactivating activity. For example, certain types of breast cancer can be controlled by blocking the estrogen receptor transactivation using the antiestrogen tamoxifen. Understanding how nuclear receptor activity such as that of AR, ER, TR4 and TR2 is regulated and how those nuclear receptors interact with each other will advance the understanding of many human diseases and clinical conditions that involve those nuclear receptors. As a consequence, new treatment options, new drug screening methods and new diagnostic tools will emerge.

BRIEF SUMMARY OF THE INVENTION

[0006] The present invention is summarized in that androgen receptor and TR4 orphan receptor can repress each other's transactivation activity by heterodimerizing with each other and therefore useful for treatment of TR4 orphan receptor- and androgen receptor-related diseases and clinical conditions.

[0007] The present invention is also summarized in that estrogen receptor and TR2 orphan receptor can repress each other's transactivation activity by heterodimerizing with each other and therefore useful for treatment of TR2 orphan receptor- and estrogen receptor-related diseases and clinical conditions.

[0008] The present invention is also summarized in that new therapeutic agents for diseases and clinical conditions related to transactivation activities of one of the androgen receptor and TR4 orphan receptor can be screened by measuring the level or transactivation activity of the other.

[0009] The present invention is also summarized in that new therapeutic agents for diseases and clinical conditions related to transactivation activities of one of the estrogen receptor and TR2 orphan receptor can be screened by measuring the level or transactivation activity of the other.

[0010] Other objects, advantages and features of the present invention will become apparent from the following specification.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 is a graphical illustration of the construction of AR deletion mutants.

Fig. 2 is a graphical presentation of data from an experiment described below. In Fig. 2, 2(A) illustrates data from an experiment analyzing TR4 interaction with AR in the mammalian two-hybrid system while Fig. 2(B) illustrates and experiment analyzing TR4 interaction with AR in modified mammalian one-hybrid system.

Fig. 3 is another graphical representation of data from an experiment described below, an experiment studying AR repression of TR4-target gene expression. In Fig. 3(A) data is illustrated related to AR repression of TR4-mediated DR4-CAT and CNTFR-15-LUC transcriptional activity, while in Fig. 3(B) the data is related to AR repression of TR4-mediated HBV gene expression.

Fig. 4 is a graphical representation of data from an experiment related to TR4 repression of AR-mediated transcriptional activity.

Fig. 5 illustrates data related to the specificity of negative regulation on AR-mediated MMTV-Luciferase activity by TR4.

Fig. 6 presents data related to the interaction between TR2 and ER in mammalian two-hybrid system. Values are presented as the mean ± SD of three independent experiments.

Fig. 7 graphically illustrates data from an experiment related to the fact that ER inhibits TR2 transcriptional activity in H1299. Values presented are the means ± SD of three independent experiments.

Fig. 8 presents data from an experiment testing the effects of TR2 on estrogen-induced ER activity in different cell lines. All values represent the mean of duplicate samples, and similar results were obtained in three independent experiments.

Fig. 9 contains data from an experiment testing the inhibitory effect of TR2 on the induction of PR mRNA (A) and protein (B) by E2 in T47D cells.

Fig. 10 is a graphical presentation of data from an experiment studying the inhibitory effect of a chimera receptor, TR2-ARp-TR2, on ER transcriptional activity. Values are the means ± SD of three independent determinations.

DETAILED DESCRIPTION OF THE INVENTION

[0011] The observation that underlies this disclosure is based on a new understanding of interactions between two sex hormone receptors and two nuclear receptors. Disclosed here is the

fact that the TR4 orphan receptor and the androgen receptor are capable of mutual co-suppression by the formation of a heterodimer. In a similar manner, mutual suppression is demonstrated between the TR2 receptor and the estrogen receptor, also by heterodimer formation. These observations make possible new approaches to the modulation of sex hormone receptors and new approaches for blocking sex hormone activity. To understand these observations, the two systems will be described in turn.

[0012] Interaction between AR and TR4.

[0013] By using techniques, like a yeast two-hybrid system described below, androgen receptor has been found to heterodimerize with TR4 orphan receptor. Such dimerization has been confirmed *in vitro* by the GST-TR4 fusion protein pull-down assay. The heterodimerization is not DHT-dependent. This heterodimerization suppresses activity of these two transcriptional factors. Thus each of these transcriptional factors is capable of suppressing the activity of the other.

[0014] The heterodimerization between the androgen receptor and TR4 was also demonstrated by an *in vivo* system using immunocytofluorescence assay, as described below. Transfected TR4 was mainly located in the nucleus while transfected AR was mainly located in the cytoplasm, in the absence of its cognate ligand DHT. However, when TR4 and AR were co-transfected, the majority of the AR was transported to the nucleus by TR4 even in the absence of DHT.

[0015] In a mammalian two-hybrid system and a mammalian one-hybrid system, as described below, the ligand binding domain of TR4 was sufficient for binding to a near full-length AR (amino acids 33-918). The binding was DHT-dependent. The difference between DHT-dependent interaction detected in the mammalian one- or two-hybrid systems and DHT-independent interaction detected in the GST pull-down and the immunocytofluorescence assays, could be due to the involvement of AF-1 ligand-independent interaction in the GST pull-down and the immunocytofluorescence assays, which used the full length of TR4 containing AF-1, vs. AF-2 ligand-dependent interaction in the mammalian one- or two-hybrid system, which used only TR4 ligand binding domain containing AF-2.

[0016] The significance of TR4/AR heterodimerization lies first in the fact that such dimerization prevents TR4 from binding to its target DNA and thus repressed TR4-mediated transactivation. This repression was observed in mammalian cells in a reporter assay, as described below. The repression is AR dose-dependent. Therefore, human diseases or clinical

conditions such as hepatitis, hepatoma and hair loss that may relate to TR4 transactivation activity may be controlled or cured through modulating AR levels in human. If a disease or clinical condition is associated with an increase of TR4 transactivation, the disease or clinical condition may be affected by modulating the AR level. If a disease or clinical condition is associated with a decrease of TR4 transactivation, the disease or clinical condition may be controlled by decreasing the AR level. Any method or agent that can change the AR level in human bodies are candidates to treat TR4-related diseases.

[0017] Since both the full length and a near full-length AR (amino acid 31 to 918) have been tested and shown to heterodimerize with TR4, it is expected that the AR LBD itself is sufficient to bind to TR4 to repress TR4's transactivation. Therefore, it is expected that any truncated form of AR that retains the LBD or any chimeric protein that contains the AR LBD may also be used to treat modulate levels of TR4 activity.

[0018] The present invention makes it possible to screen for drugs for TR4-related diseases by testing a compound's effect on AR level. If a compound can increase or decrease the level of AR in a cell, it can be selected for further testing for treatment of TR4-related diseases. The screening method can measure the AR level directly. It can also measure the AR level indirectly, for example, through any reporter system that measures AR transactivation. Examples of such reporter systems are described below.

[0019] The significance of TR4/AR heterodimerization lies secondly in the fact that it also represses AR-mediated transactivation. When cells were treated with DHT, AR was induced to transactivate several downstream genes. Overexpression of TR4 in the same cells could block the AR-induced expression of these downstream genes. Therefore, the present invention is also directed at new treatment strategies for AR-related diseases such as prostate cancer by modulating TR4 levels to enhance or repress AR's transactivation activity. If a disease or clinical condition is associated with an increase of AR-mediated transactivation, the disease or clinical condition may be cured or controlled by increasing the TR4 level. If a disease or clinical condition is associated with a decrease of AR-mediated transactivation, the disease or clinical condition may be cured or controlled by decreasing the TR4 level. The full length TR4 can repress AR's transactivation activity. Any method or agent that can change TR4 levels in human bodies are candidates for treating AR-related diseases. Since the LBD of TR4 could bind to AR as well, any truncated form of TR4 that retains the LBD or any chimeric protein that contains the TR4 LBD may also be used when treatment requires a repression of AR transactivation.

[0020] Although only DHT-induced AR transactivation was tested to show that TR4 could repress AR transactivation, it is expected that AR transactivation induced in any manner, including that by constitutively active AR, can be repressed by TR4.

[0021] The present invention makes it possible to screen for drugs for AR-related diseases by testing a compound's effect on TR4 level. If a compound can increase or decrease the level of TR4 in a cell, then it can be selected for further testing for treatment of AR-related diseases. The screening method can measure TR4 level directly. It can also measure TR4 level indirectly, for example, through any reporter system that measures TR4 transactivation. Examples of such reporter systems are described below.

[0022] Interaction between ER and TR2.

[0023] Using an *in vitro* GST pull-down assay and an *in vivo* mammalian two-hybrid system, ER and TR2 were found to heterodimerize. In the *in vitro* GST pull-down assay, it was further found that the LBD (aa 499-595) or the F domain (aa 552-595) of ER could also bind to TR2. In the *in vivo* mammalian two-hybrid system, E2 could promote the dimerization between ER LBD (aa 282-595) and TR2.

[0024] The significance of TR2/ER dimerization lies first in the fact that it prevented ER from binding to its target DNA and thus repressed ER transactivation. TR2 LBD was sufficient to prevent ER from binding to its target DNA. When cells were treated with E2, ER was induced to transactivate several downstream genes. Overexpression of TR2 in the same cells could block the ER-induced expression of these downstream genes in a dose-dependent manner. Therefore, the present invention is also directed at new treatment strategies for ER-related diseases such as breast cancer by modulating TR2 levels to enhance or repress ER's transactivation activity. If a disease or clinical condition is associated with an increase of ER-mediated transactivation, the disease or clinical condition may be cured or controlled by increasing the TR2 level. If a disease or clinical condition is associated with a decrease of ER-mediated transactivation, the disease or clinical condition may be cured or controlled by decreasing the TR2 level. The full length TR2 could repress AR's transactivation activity. Any method or agent that can increase or decrease TR2 levels in human bodies are candidates for treating ER-related diseases. Since the LBD of TR2 could prevent ER from binding to its target DNA as well, any truncated form of TR2 that retains the LBD or any chimeric protein that contains the TR2 LBD may also be used when treatment requires repressing ER's transactivation activity.

[0025] Although only E2-induced ER transactivation was tested to show that TR2 could repress ER transactivation, it is expected that ER transactivation induced in any manner, including that by constitutively active ER, can be repressed by TR2.

[0026] The present invention also makes it possible to screen for drugs for ER-related diseases by testing a compound's effect on TR2 level. If a compound can increase or decrease the level of TR2 in a cell, then it can be selected for further testing for treatment of ER-related diseases. The screening method can measure TR2 level directly. It can also measure TR2 level indirectly, for example, through any reporter system that measures the increase or decrease of TR2 transactivation. Examples of such reporter systems are described below.

[0027] The significance of TR2/ER heterodimerization lies second in the fact that it repressed TR2 transactivation activity in an ER dose dependent manner. Therefore, human clinical conditions such as hair loss that may relate to TR2 transactivation activity may be controlled through modulating ER levels in human. If a disease or clinical condition is associated with an increase of TR2-mediated transactivation, the disease or clinical condition may be cured or controlled by increasing the ER level. If a disease or clinical condition is associated with a decrease of TR2-mediated transactivation, the disease or clinical condition may be cured or controlled by decreasing the ER level. Any method or agent that can change ER levels in human bodies are treatment candidates.

[0028] Since ER LBD could also bind to TR2, any truncated form of ER that retains the LBD or any chimeric protein that contains the ER LBD are also potential treatment candidates when treatment requires repressing TR2 transactivation.

[0029] The present invention also makes it possible to screen for drugs for TR2-related diseases by testing a compound's effect on ER level. If a compound can increase or decrease the level of ER in a cell, then it can be selected for further testing for treatment of TR2-related diseases. The screening method can measure ER level directly. It can also measure ER level indirectly, for example, through any reporter system that measures the increase or decrease of ER transactivation. Examples of such reporter systems are described below.

[0030] In addition, TR2 can form heterodimers with either TR4 or AR as well. It is expected that co-repression exists in these two dimers as well.

EXAMPLES

GST pull-down assay

[0031] GST-TR4 fusion protein and GST control protein were purified as instructed by the manufacturer (Pharmacia). Five ml *in vitro* translated ^{35}S methionine-labeled proteins were used to perform pull-down assay as described previously. Cacailles, et al., *EMBO* 14, 3741-3751 (1995).

Immunocytofluorescence

[0032] DU145 cells were seeded on two-well Lab Tek Chamber slides (Nalge Nunc International) 18 h before transfection. One to two mg of DNA per 10^5 cells was transfected by the FuGENE™ 6 transfection reagent (Boehringer-Mannheim). Transfected cells were treated with 100 nM DHT. Immunostaining was performed by incubating with anti-AR polyclonal antibody (NH27), anti-TR4 monoclonal antibody (#15), and anti-ERα monoclonal antibody (C-314, Santa Cruz), followed by incubation with either fluorescein-conjugated goat anti-rabbit or anti-mouse antibodies (ICN Pharmaceuticals, Inc.). Yang, et al., *Proc. Natl. Acad. Sci.*, USA 94, 13075-13080 (1997). The slides were photographed under 100-fold magnification using confocal microscopy.

Transient Transfection

[0033] Cells were routinely maintained in DMEM with 10% heat-inactivated fetal bovine serum. The cells were transfected using a modified calcium phosphate precipitation method, Mizokami, & Chang, *J. Biol. Chem.* 269, 25655-25659 (1994), or SuperFect (Qiagen). To normalize the transfection efficiency, the (3-galactosidase expression ver and pRL-TK were co-transfected in CAT assay and in Dual-luciferase reporter assay system (Promega), respectively.

Electrophoretic Mobility Shift Assay

[0034] EMSA was performed as described previously. Lee, et al., *J. Biol. Chem.* 272, 12215-12220 (1997). Briefly, the reaction was performed by incubating the ^{32}P-end labeled DR1 probe with *in vitro* translated TR4 (1 ml) with or without an increasing amount of AR (1, 2, 4 ml). The EMSA incubation buffer is 10 mM HEPES, pH 7.9, 2% (v/v) glycerol, 100 mM KCl, 1mM EDTA, 5 mM MgCl,, and 1 mM DTT. For the antibody supershifted analysis, 1 ml of monoclonal anti-TR4 antibody (#15) was added to the reaction. DNA-protein complexes were resolved on a *5%* native gel. The radioactive gel was analyzed by autoradiography.

Northern Blotting Analysis

[0035] Total RNA from the DHT-treated transfected LNCaP cells was prepared by the ultracentrifugation method as described. Lee, et al., *J. Biol. Chem.* 273, 13437-13443 (1998). The probe was obtained from exon 3 of PSA gene and labeled with α-^{32}P dCTP.

[0036] TR4 Interacts with AR both *In Vitro* and *In Vivo.*

[0037] Using a GAL4-TR4 fusion protein as bait, we were able to use the yeast two-hybrid system to isolate several potential TR4 associated proteins. Sequence analysis confirmed that some of the candidates, such as AR and the TR2 orphan receptor, could physically interact with TR4. GST-TR4 fusion protein pull-down assay was performed to further confirm the result. GST-TR4 fusion protein and GST control protein were purified as instructed by the manufacturer (Pharmacia). Five µl of *in vitro* translated ^{35}S methionine-labeled AR, TR2, and RXRα were incubated with GST-TR4 or GST bound to glutathione-Sepharose beads in a pull down assay. TR4 was found to physically interact with AR and TR2 orphan receptor. In contrast, there was no interaction between TR4 and RXR, another member of the steroid receptor superfamily.

[0038] AR was further characterized due to its profound effects on many androgen-related diseases. To map more precisely the regions in AR that can interact with TR4, various AR deletion mutants, AR-N, AR-D, and AR-L were *in vitro* translated and incubated with GST-TR4 in a pull-down assay. Figure 1 illustrates the deletions associated with each of those deletion mutants. The pull-down complex was loaded onto an 8% or 15% polyacrylamide gel and visualized by autoradiography. TR4 was found to interact with three *in vitro*-translated ^{35}S methionine AR deletion constructs, the N-terminal of AR (AR-N), the DBD of AR (AR-D), and the LBD of AR (AR-L). These results agreed with previous reports that coregulators were able to interact with both N-terminal and C-terminal domains of steroid receptors. Gelman, et al., *J. Biol. Chem.* 274, 7681-7688 (1999).

[0039] An immunocytofluorescence assay was then applied to determine the subcellular localization of the AR and TR4 in DU145 cells. DU145 cells were seeded on two-well Lab Tek Chamber slides (Nalge Nunc International) 18 hours before transfection. One to two µg of DNA per 10^5 cells was transfected either with AR (unliganded or liganded), TR4, or ER alone or in combination with the FuGENE™6 transfection reagent (Boehringer-Mannheim). After 24 hours transfection, cells were treated with 100 nM DHT or ethanol. Immunostaining was performed by incubation with the anti-AR polyclonal antibody, anti-TR4 monoclonal antibody, or anti-ERα monoclonal antibody, followed by incubation with either fluorescein-conjugated goat anti-rabbit

or anti-mouse antibodies (ICN Pharmaceuticals, Inc.). We found that unliganded AR was located mainly in the cytoplasm. The AR signal moved to the nucleus in the presence of its cognate ligand, DHT. These data agreed with a previous report shown in COS cells. Simental, et al., *J. Biol. Chem.* 266, 510-518 (1991). In contrast, TR4 was detected as a nuclear protein as was ER even in the absence of exogenous ligand.

[0040] Interestingly, when AR and TR4 were co-transfected into DUI4S cells, the majority of the AR signal could be detected together with TR4 signal in the nucleus, even in the absence of DHT. This data indicates that unliganded cytosolic AR moves into the nucleus once it is coexpressed with TR4 in DU145 cells. In contrast, when AR and ER were co-transfected into DU145 cells, the AR signal still remained mainly in the cytoplasm in a manner similar to that found when AR was transfected alone. The observation that unliganded AR can translocate into the nucleus in the presence of TR4 provides strong *in vivo* evidence that AR interacts specifically with TR4.

[0041] The ability of TR4 to interact with AR was further evaluated by the mammalian two-hybrid system assay. A near full-length human AR (amino acids 33-918) was fused to the transcriptional activator VPL6 (VP16-AR) and then co-transfected with GAL4-DBD fused with TR4 LBD (GAL4-TR4E) and a GAL4-responsive luciferase reporter (PG5-Luc) in H1299 cells. More specifically, 3.5 μg of PG5-Luc, the luciferase reporter gene containing five copies of GAL-DBD binding sites, was cotransfected with two fusion proteins, GAL4-TR4E, and VP16-AR. After 16-18 hours transfection, 1 nM DHT was added and ethanol was used in control groups. After 24 hours treatment, cells were harvested for Dual Luciferase Assay. The results, illustrated in Fig. 2A, revealed that either parental vector (pCMV-GAL4 or pCMV-VPI6), VP16-AR, or GAL4-TR4E alone, showed a low background in the absence or presence of 1 nM DHT. Upon co-transfection of VPI6-AR and GAL4-TR4E, a significant induction was only observed when we added 1 nM DHT (see lane 10), thus indicating that DHT could promote the interaction between GAL4-TR4E and VPL6-AR.

[0042] We used a modified mammalian one-hybrid system to avoid the possibility that the DHT-dependent interaction between AR and TR4 is due to artificial conformational changes created by the VPI6-AR fusion protein in the mammalian two-hybrid system. A full-length AR(pSG5AR) was co-transfected with GAL4-TR4E and PG5-Luc reporter in H1299 cells. More specifically, 3.5 μg of PG5-Luc and 3 μg of GAL4-TR4E were co-transfected in the presence of 1 μg of pSG5AR, pSG5GR, pSG5PR, or pSG5ER. Cells were treated as indicated in Fig. 2B.

Transfection was performed by a modified calcium phosphate precipitation method. The pRL-TK plasmid was co-transfected for normalization of transfection efficiency. As shown in Fig. 2B, transfection of pSG5AR alone showed only marginal DHT-dependent transcriptional induction (lanes 2, 3, and 4), whereas, co-transfection of pSG5AR and GAL4-TR4E showed a significant (20-40 fold) induction in the presence of 1-10 nM DHT (lanes 6 and 7). In contrast, no induction was observed when we replaced AR with other activated steroid receptors, such as glucocorticoid receptor (GR), progesterone receptor (PR), or estrogen receptor (ER) (lanes 10, 11, and 12). Moreover, addition of 1 μM of antiandrogens, such as hydroxyflutamide or RU58841, could abolish the DHT-enhanced interaction between AR and TR4 (lanes 8 and 9).

[0043] The difference between DHT-dependent interaction detected in the mammalian one- or two-hybrid systems and DHT-independent interaction detected in the GST pull-down and the immunocytofluorescence assays, could be due to the involvement of AF-1 ligand-independent interaction in the GST pull-down and the immunocytofluorescence assays, which used the full length of TR4 containing AF-1, vs. AF-2 ligand-dependent interaction in the mammalian one- or two-hybrid system, which used only TR4 ligand binding domain containing AF-2. Taken together, data from the GST pull down assay, the immunocytofluorescence assay, and the experiments done with the mammalian one- and two-hybrid system provide strong evidence that AR and TR4 can interact in the absence or presence of DHT.

AR Represses TR4-Mediated Transactivation.

[0044] The potential effects on transactivation by the AR-TR4 heterodimer formation was then tested through use of a reporter assay: full-length AR and TR4 in eukaryotic expression vectors (pSG5AR and pCMXTR4) were co-transfected with a CAT reporter containing a TR4-response element (DR4-TK-CAT), Lee, et al., *J. Biol. Chem.* 272, 12215-12220 (1997), in H1299 cells. More specifically, we co-transfected 500 ng of reporter plasmids (DR4-CAT and CNTFR-15-LUC) with 200 ng of pCMX-TR4 and increasing amounts of pCMV-AR (200, 600, and 1,200 ng), pSG5GR (1,200 ng), or pSG5 PR (1,200 ng) using the SuperFect transfection kit (Qiagen). As shown in Fig. 3A, the CAT activity induced by pCMXTR4 could be repressed significantly, in a dose-dependent manner by co-transfection of pSG5AR in the absence or presence of DHT. This repression of TR4 transactivation is AR specific, as other activated steroid receptors, such as GR or PR, have no suppressive effects (Fig. 3A, lanes 9-10). Similar results were obtained when we replaced the DR4-TK-CAT reporter with DR1-CNTFR-15-LUC, another TR4 response element (Fig. 3A). Young, et al., *J. Biol. Chem.* 272, 3109-3116 (1997).

[0045] We also investigated another TR4 potential target gene, which is located in the hepatitis B virus (HBV) enhancer II region (-34 to -7) containing a classic DR1 motif. Breidbart, et al., *Pediatric Res.* 34, 300-302 (1993). The reporter plasmid CpFL(4)-LUC, which contains the HBV core promoter (Cp) sequence located between -34 and -7 (nucleotide coordinates 1751 and 1778 derived from the Genbank database) was shown above in Fig. 3B. The arrows indicate the DR1 motif in HBV core promoter. HepG2 cells were co-transfected with 1.5 μg CpFL(4)-LUC reporter and 0.5 μg pCMX-TR4, with increasing amounts of pCMV-AR (0.5, 2.5, and 5 μg) by modified calcium phosphate precipitation method. The relative reporter gene activities were compared to the CAT activities (or luciferase activities) with vector alone. To normalize the transfection efficiency, the β-galactosidase expression vector and pRL-TK were co-transfected in the CAT assay and in the Dual-luciferase reporter assay system (Promega), respectively. As shown in Fig. 3B, TR4 can induce CpFL(4)-LUC activity, which is significantly decreased by co-transfection of AR with TR4 in a dose-dependent manner. This finding suggested that AR could regulate HBV gene expression through protein-protein interaction.

AR Prevents TR4 from Binding to Its Target DNA.

[0046] EMSA using ^{32}P labeled DR1-TR4RE as a probe were applied to further dissect the mechanism of how AR repressed the TR4-mediated transactivation. One μl of *in vitro* translated TR4 protein was incubated with increasing amounts of *in vitro* translated AR (1 μl, 2 μl, and 4 μl) in EMSA reaction buffer (10 mM HEPES pH 7.9, 2% (v/v) glycerol, 100 mM KCl, 1mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT) for 15 min. ^{32}P-end labeled DR1 was added into the protein mixture and incubated for 15 min before loading. For the antibody supershift assay, 1 μ1 of monoclonal anti-TR4 antibody was added to the reaction and applied to a 5% native polyacrylamide gel. The radioactive gel was analyzed by autoradiography. The specific TR4-DR1 band was decreased with the addition of increasing amounts of AR. Furthermore, the intensity of the supershifted band formed by the adding an anti-TR4 monoclonal antibody (McAb) to the TR4-DR1 complex, was also decreased with the addition of increasing amounts of AR. Together, these results suggested that AR might be able to repress TR4-mediated transactivation by preventing TR4 from binding to its target DNA. As there is no extra supershifted band formed upon adding AR to TR4-DR1 complex, our data may also rule out the possibility of the formation of a transcriptional inactivated TR4-AR-DR1 complex.

TR4 Represses AR Target Gene Activation Both *In Vitro* and *Vivo.*

[0047] Like TR4, AR itself acts as a transcription factor to activate many androgen target genes. We were interested in investigating the potential negative-regulatory effects of TR4 on AR-mediated transactivation. 500 ng of MMTV-Luc (Fig. 4A), or PSA-Luc (Fig. 4B), were co-transfected with 40 ng pCMV-AR (lane 2-3) with increasing amounts of pCMX-TR4 (as indicated in Figure 4). After 24 hours transfection, cells were treated with 10 nM of DHT. After 16-18 hours incubation, cells were harvested for Dual-luciferase reporter assay. As expected, in COS, H1299, and CHO cells, AR activated MMTV luciferase activity in a DHT-dependent manner (Fig. 4A, lanes 2 and 3), which could then be repressed by the addition of TR4 (lanes 6 to 8). TR4 by itself has no effect on MMTV luciferase activity in the absence or presence of 10 nM DHT (lanes 4 and 5). Similar suppression effects also occurred when we replaced the MMTV-luciferase reporter with the PSA-luciferase reporter, another AR target gene (Fig. 4B).

[0048] To rule out the potential artificial effects linked to transfected reporter assays, the expression of endogenous prostate specific antigen (PSA, an androgen target that is widely used as a marker for prostate cancer progression) in LNCaP cells was measured by Northern blot analysis. Total RNA (25 μg) from LNCaP cells, which were transfected with either pCMX-TR4 or pCMX vector using SuperFect (Qiagen), was applied into a formamide RNA gel, then transferred onto a Nylon membrane, and then hybridized with a ^{32}P-PSA gene fragment from the exon 3. β-actin was used as an internal control. As shown in Fig. 4C, the expression of PSA transcript was induced about 2.5 fold after 24 hours of DHT treatment (lane 3 *vs.* 4). Addition of TR4 can clearly repress the expression of endogenous PSA transcript in either the absence (lane 1 *vs.* 3) or presence of 10 nM DHT (lane 2 *vs.* 4). The level of secreted PSA protein in the medium measured by ELISA, also confirmed our conclusion (data not shown). This *in vivo* TR4-mediated suppressive effect strongly supports the above reporter assays and demonstrates that TR4 may function as a repressor to negatively-regulate expression of AR target genes expression.

TR4 Represses AR-Mediated Transactivation Specifically.

[0049] As GR and PR can also induce MMTV-luciferase reporter, Beato, M., *Cell* 56, 335-344 (1989), we were interested in determining if TR4 could also repress OR- or PR-mediated transactivation. Three μg of MMTV-Luc was co-transfected with 4 μg of pCMX-TR4 in the presence of 1 μg of pSG5AR, pSG5GR, or pSG5PR by modified calcium-phosphate method. After 24 hours transfection, the cells were treated with 10 nM of synthetic steroids (DHT, dexamethasone, and progesterone). Dual-luciferase reporter assays were performed. PRL-

TK was used to normalize the transfection efficiency. As shown in Fig. 5, while AR, GR, and PR could induce MMTV-luciferase activity in the presence of their respective ligands in H1299 cells, co-transfection of TR4 could only repress AR-mediated transactivation. Similar results were observed when we repeated the same experiments of AR-mediated transactivation in DU145 cells. To our knowledge, TR4 represents the first receptor that can heterodimerize with AR with subsequent down-regulation of AR transactivation.

[0050] Previous reports suggested that RXR could function as a coactivator through heterodimer formation with the receptors for Vitamin D (VDR), thyroid hormone (TR), and peroxisome proliferator (PPAR). Yu et al., *Cell* 67, 1251-1266 (1991); Kliewer et al., *Nature* 355, 446-449 (1992); Kliewer, et al., *Nature* 358, 771-774 (1992); Zhang, et al., *Nature* 355, 441-446 (1992). The reverse repression effects of VDR, TR, and PPAR on RXR target genes, however, remain unknown. Our bi-directional repression effects through the AR and TR4 heterodimerization, therefore, represent a new mechanism in the steroid receptor superfamily signaling pathway. The physiological significance of the AR-TR4 heterodimer is further supported by the similar expression pattern of both receptors in many tissues, such as the testis, hypothalamus, and prostate. Chang, et al., *Proc. Natl. Acad. Sci USA*. 91, 6040-6044 (1994); Lee, et al., *J. Biol. Chem.* 274, 16198-16205 (1999); Chang, et al., *Gene Expression* 5, 97-126 (1995).

[0051] Two potential impacts of these new findings are significant. First, the role of AR in the modulation of androgen target genes may be expanded. In addition to activation of classic androgen target genes containing androgen response elements (GGA/TACAnnnTGTTCT), AR may also signal through heterodimerization with TR4, resulting in the repression of various TR4 target genes, which contain a consensus response element (AGGTCA) in a DR orientation ($AGGTCA(n)_xAGGTCA$, x = 0-6). Data from our gel shift assays showed that the binding preference of TR4 for the natural TR4RE identified in various target genes, was in the order of DR1 (CRBII-TR4RE) > DR2 (SV40-TR4RE) > DR4 (TRE-TR4RE) > DR5 (RAREβ-TR4RE) > DR3 (VDRE-TR4RE), with the IC_{50} varying widely from 0.023 ng to 2.0 ng. Lee, et al., *J. Biol. Chem.* 273, 13437-13443 (1998); Lee, et al., *J. Biol. Chem.* 272, 12215-12220 (1997); Lee et al., *J. Biol. Chem.* 274, 16198-16205 (1999); Lee, et al., *J. Biol. Chem.* 270, 30129-30133 (1995). Among these TR4 target genes that could be suppressed by AR, HBV suppression might be especially interesting as it provides the first evidence that AR may play a suppressive role in the HBV expression. Whether this may contribute to the male-preference Hepatitis B or hepatoma

will be an interesting topic for future study. Secondly, we have demonstrated that the classic androgen-signaling pathway (A→AR→ARE) can be influenced by TR4. This not only represents the first mechanism to distinguish between receptors (AR, GR, and PR) that share the same hormone response elements (found in MMTV or other target genes), but also provides a new potential target through which to block the androgen action. The long-term impact of these two new events may be in providing us another approach in the design of the new generation of drugs with androgenic or antiandrogenic activity with which to treat androgen-related diseases.

Chemicals and Plasmids

[0052] [^{14}C] Chloramphenical was obtained from Amersham Corp (Arlington Heights, IL 60005). Acetyl coenzyme A was purchased from Pharmacia Biotech Inc (Piscataway, NJ 08854). 17β-estradiol (E2) was purchased from Sigma Chemical Co (St. Louis, MO 63178). The in vitro transcription/translation (TNT) coupled reticulocyte lysate system was purchased from Promega (Madison, WI 53711).

[0053] Human full-length ER was inserted into the EcoR I site of pSG5 to produce pSG5-ER. The pCMV-TR2, pCMX-VPI6-TR2, and pGEX-3x-TR2 were constructed by inserting full-length TR2 fragments to individual vectors. The GAL4-ER, amino acid (aa) 282-595, plasmid was a kind gift from Dr. Hinrich Gronemeyer. Constructs corresponding to GST-ER fragments were made using the vector pGEX series (Pharmacia, Piscataway, NJ 08854). Inserts of the ER fragments were released from the pSG5-ER and generated by the following strategies: GST-ER-N-terminal (GST-ER-N), the BamHI-MscI fragment of ER into the pGEX-3x SmaI-RcoRI site; GST-ER-DNA Binding Domain (GST-ER-DBD), the HindIII-PstI fragment into the pGEX-2T SmaI site; GST-ER-Ligand Binding Domain (GST-ER-LBD), the PstI-EcoRI fragment into the pGEX-2T SmaI-EcoRI site; and GST-ER-F Domain (GST-ER-F) the HhaI-EcoRI fragment into the pGEX-3x SmaI site. All plasmids were verified by restriction enzyme analysis and DNA sequencing.

Cell Culture

[0054] H1299 human lung cancer cells and PC-3 human prostate cancer cells were maintained in DMEM containing 5% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/ml streptomycin sulfate at 5% C02 at 37°C. T47D human breast cancer cells were maintained in RPMI 1640 medium containing 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 5% C02 at 37°C.

Transfections and Reporter Gene Expression Assays

[0055] Transfections and chloramphenicol acetyltransferase (CAT) assays were performed as described previously. Yeh, & Chang, *Proc. Natl. Acad. Sci.* 93, 5517-5521 (1996). Briefly, 4 x 10^5 cells were plated in 60-mm dishes and cultured for 24 h, and then the medium was changed to phenol red free DMEM with *5%* charcoal-stripped FCS 2 h before transfection. The cells were co-transfected with TR2 and/or ER expression plasmids with 2μg of TR2-TK-CAT reporter or 1 μg of ERE-CAT reporter gene plasmid, as indicated in the Figures, by using the calcium phosphate precipitation method. A β-galactosidase expression plasmid, pCMV-β-gal, was transfected in all transfections as an internal control for normalizing transfection efficiency. The total amount of DNA was adjusted up to 10.5 μg with parent vectors, pSG5 or pCMV in each transcriptional activity assay. After 24 h transfection, the medium was changed again and the cells were treated with 10^{-8} M E2 for another 24 h. The cells were then harvested and whole cell extracts were used for CAT assay. The CAT activity was quantitated by PhosphorImager (Molecular Dynamics). Data are presented as means ± S.D. of at least three independent experiments

GST Pull-down Assay

[0056] Fusion proteins of GST-TR2, GST-ER segments and GST were obtained by transforming expressing plasmids into BL21(DE3)pLysS competent bacteria followed with 2-hour IPTG induction. GST-proteins were then purified by mixing Glutathione-Sepharose™ 4B (Pharmacia) into bacteria lysates on a rotating disk at 4°C for 40 mm followed by washing with lmL NENT buffer (20 mM Tris-HCL (pH 8.0), 100 mM NaCl, 1 mM EDTA, 6 mM $MgCl_2$, 1 mM Dithiothretiol, 8% Glycerol, 1 mM PMSF and 0.5% (v/v) NP-40). The ER, AR, RXRα, and TR2 proteins labeled with [^{35}S] were generated *in vitro* by using the TNT reticulocyte lysate system (Promega). For the *in vitro* interaction, the glutathione-Sepharose bound GST-proteins were resuspended with 100 μl of interaction buffer (20 mM HEPES pH 7.9, 150 mM KCl, 5 mM $MgCI_2$, 0.5 mM EDTA, 0.5 mM Dithiothretiol, 0.1% (v/v) NP-40, 0.1% (w/v) BSA and 1 mM PMSF) and mixed with 5 μl of rotating disk at 4°C for 3h. After extensive washes with NENT buffer, the bound proteins were separated on an SDS/8% PAGE and visualized by using autoradiography.

Mammalian Two-Hybrid Assay

[0057] Transfections were performed using the calcium-phosphate precipitation method described above. H1299 cells or PC-3 cells were transiently co-transfected with 3 μg of a GAL4-

ER expression plasmid, 3 μg of a VPI6-TR2 expression plasmid, and 3 μg pG5-CAT reporter plasmid. CAT assays were performed as described above. 1 μg of a β-galactosidase expression plasmid, pCMV-β-gal, was used as an internal control. The total amount of DNA was adjusted to 10.5 μg with parent vectors in all transcriptional activity assays.

Electrophoretic Mobility Shift Assay (EMSA)

[0058] EMSA was carried out as described previously with some modification. Lee,et al., *J. Biol. Chem.* 274, 13437-13443 (1998). 0.1 μg of Double-stranded oligonucleotide ERE primers were end-labeled with 5 μl of [γ-^{32}P]ATP (DuPont NEN) by using T4 polynucleotide kinase. *In vitro* translated proteins, 1 μl of ER protein and 2 μl of TR2, were incubated with the 0.1 ng of [^{32}P]-labeled ERE probe (4 x 10^8 dpm/μg) in 20 μl of EMSA binding buffer (50 mM HEPES pH 7.9, 500 mM KCl, 5 mM Dithiothretiol, 2.5 mM EDTA, 12.5 mM $MgCl_2$, 30% glycerol, 10% Ficoll) on ice for 30 min. For the competition reactions, 100 ng unlabeled ERE oligonucleotides were mixed with the labeled probe prior to addition to the reactions. For the antibody supershift assays, 1 μl of the monoclonal anti-ERα antibody (C-314, Santa Cruz) was added into the reactions for additional 30 min. The protein-DNA complex was analyzed on a 5% polyacrylamide native gel containing 2.5% glycerol in 0.5 X TBE buffer (45 mM Tris borate, 1 mM EDTA).

Western Blot

[0059] The method was used as previously described, Lee & Chang, (1996)J. Biol. Chem. 271, 10405-10412, with some modifications. Briefly, 5 μg of pCMV-TR2 was transiently transfected into T47D cells, and lysed in RIPA buffer (10 mM sodium phosphate, pH 7.0, 150 mM NaCL, 2 mM EDTA, 1 % (w/v) Nonidet P-40, 0.1 % (w/v) SDS, 1% (w/v) sodium deoxycholate) with freshly adding proteinase inhibitors. The soluble protein was quantified using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories) and 50 μg soluble proteins were loaded onto SDS/10% PAGE and then transferred to Immobion-P transfer membrane (Millipore). After the blocking reaction overnight, the membrane was incubated with rat anti-PR polyclonal antibody (H-190, Santa Cruz) in PBS(-) containing 0.1% Skim milk for 2 h at room temperature. The membrane was washed and then incubated in 15 μCi [^{125}I] of protein-A (DuPont NEN)/30 ml of PBS(-) containing 0.1% Skim milk for 1 h at room temperature. The western blots were autoradiographed and quantitated by using PhosphorImager.

Other Methods

[0060] RNA isolation and Northern blot analysis were performed as previously described. Young, et al., *J. Biol. Chem.* 273, 20877-20885 (1998).

Interaction between ER and TR2.

[0061] The *in vitro* GST pull-down assay was applied to examine the interaction between the TR2 and ER. *In vitro*-translated, [^{35}S]-labeled ER, AR, and RXRα proteins were incubated with GST or GST-TR2 bound on glutathione-Sepharose beads. After extensive washing, proteins were separated on an SDS/8% PAGE and visualized by using autoradiography. *In vitro* translated [^{35}S]-ER and the androgen receptor (AR), but not retinoic A receptor α (RXRα), can interact well with the GST-TR2 that is bound to the glutathione-Sepharose beads, suggesting the TR2 may be able to form a heterodimer with the ER. *In vitro*-translated, [^{35}S]-labeled TR2 protein was incubated with GST, GST-ER-N (aa 1-165 of ER), GST-ER-DBD (aa 123-340 of ER), GST-ER-LBD (aa 499-595 of ER), or GST-ER-F (aa 552-595 of ER) in the absence or presence of 1 μM E2. The partial LBD, (aa 499-595) or the F domain (aa 552-595) of the ER, but not the N-terminal domain (aa 1-165) or DBD (aa 123-340) of the ER, can interact with the [^{35}S]-TR2 in the presence of 1 μM E2.

[0062] The *in vivo* mammalian two-hybrid system was applied to further confirm the interaction between the ER and TR2. A full-length TR2 was fused to the transcriptional activator VP16 (VPI6-TR2) and then co-transfected with GAL4-DBD fused with ER-LBD (GAL4-ER, aa 282 to 595) and a GAL4-responsive CAT reporter (pG5-CAT) in H1299 cells. More specifically, H1299 cells and PC-3 cells were transiently co-transfected with 3 μg of pSG5-CAT reporter plasmid, 3 μg of GAL4 or GAL4-ER expression plasmid, and 3 μg of VPI6 or VP16-TR2 expression-plasmid. Interaction was estimated by determining, CAT activity levels in the presence or absence of 10^{8} M E2. Cells were also transfected with a β-galactosidase expression plasmid, pCMV-β-ga1, as an internal control for transfection efficiency. As shown in Fig. 6, co-transfection of the parental vector pCMX-GAL4 with pCMX-VPI6 or VP16-TR2 results in a low background in the presence or absence of 10 nM E2. While co-transfection of the pCMX-VP16 with GAL4-ER showed some self-activation in the presence of 10 nM E2, a significant induction of CAT activity was observed only when cells were co-transfected with the VP16-TR2 and GAL4-ER in the presence of 10 nM E2, indicating that the E2 could promote the interaction between the GAL4-ER and VP16-TR2. Similar results were obtained when we replaced the H1299 cells with prostate PC-3 cells (Fig. 6). Taken together, results from the *in vitro* GST pull-

down and the *in vivo* mammalian two-hybrid assays provide strong evidence that the ER and TR2 can interact with each other in the presence of E2.

ER Functions as Repressor to Repress the TR2-mediated Transactivation.

[0063] The CAT reporter assay was used to study the potential consequence of the formation of heterodimers between the TR2 and ER. Full-length ER and TR2 in eukaryotic expression vectors (pSG5-ER and pCMV-TR2) were co-transfected with a CAT reporter containing a TR2-response element (DR4-TK-CAT), Lee, et al., *J. Biol. Chem.* 272, 12215-12220 (1997), in H1299 cells. More specifically, H1299 cells were transiently cotransfected with 2 μg of DR4-TK-CAT reporter plasmid and 3 μg of TR2 expression plasmid together with increasing amounts of the ER expression vector. CAT activity was analyzed in the absence (Fig. 7A) or presence (Fig. 7B) of 10^{-8} M E2. Cells were also transfected with internal control reporter plasmid, pCMV-β-gal, as an internal control for transfection efficiency. Luciferase activity was then analyzed following manufacturer's instructions (Promega). As shown in Fig. 7A and 7B, the CAT activity induced by the pCMV-TR2 could be repressed in a dose-dependent manner by co-transfection of the pSG5-ER in the absence (Fig. 7A) or presence (Fig. 7B) of E2. Similar results were obtained when we replaced the DR4-TK-CAT reporter with DR1-HBV-LUC, another TR2 response element (Fig. 7C). Other steroid receptors, such as PR and glucocorticoid receptor (GR), show no suppressive effec. The data therefore suggests that the ER is able to function as a repressor to repress the TR2-mediated transactivation in H1299 cells.

Suppression of ER Target Gene Expression by TR2.

[0064] As the TR2 was able to suppress several genes that are regulated by the vitamin D receptor, thyroid hormone receptor, and retinoic acid receptor, Lin, et al., *J. Biol. Chem.* 270,30121-30128 (1995), Lee, et al., *J. Biol. Chem.* 272, 12215-12220 (1997), Lee, et al., *J. Biol. Chem.* 274, 13437-13443 (1998), we were interested in determining the potential reverse effects of the TR2 on the ER transactivation. PC-3 cells and H1299 cells were co-transfected with 1 μg of ERE-CAT reporter plasmid and 1 μg of ER expression plasmid together with increasing amounts of the TR2 expression vector. Relative CAT activity was determined in the absence or presence of 10^{-8} M E2. As shown in Fig. 8A, the ERE-CAT activity was induced by transfection of the pSG5-ER in the presence of 10 nM E2 in PC-3 cells (lane 1 *vs* 2). Addition of the TR2 resulted in the suppression of ER transactivation in a dose-dependent manner (lane 2 *vs* 3-5). Similar suppression effects also occurred when we replaced the PC-3 cells with H1299 cells (Fig 8A).

[0065] To eliminate the potential artificial effects caused by overexpression of exogenous ER, we chose to use T47D cells as a model to test the TR2 suppression effect on the endogenous ER-mediated transactivation. T47D cells were transfected with 1 μg of ERE-CAT reporter plasmid together with increasing amounts of the TR2 expression plasmid. The CAT-activity was observed with the endogenous ER in the absence or presence of 10^{-8} M E2. All of the cells also were transfected with a β-galactosidase expression plasmid, pCMV-β-gal to normalize the transfection efficiency. As expected, the TR2 can still repress the endogenous ER-mediated ERE-CAT activity (Fig. 8B). For a control, we also tested the potential suppression effect of the TR2 on other steroid receptors-mediated transactivation, such as PR or GR. H1299 cells were co-transfected with 1 μg of MMTV-CAT reporter plasmid and 1 μg of PR or GR expression plasmid together with increasing amounts of the TR2 expression vector. Relative CAT activity was observed in the absence or presence of 10^{-8} M progesterone or glucocorticoid, respectively. All of the cells also were transfected with a β-galactosidase expression plasmid, pCMV-β-gal to normalize the transfection efficiency. We found that TR2 has little effect on the PR- or GR-mediated transactivation (Fig. 8C). Taken together, data from Fig. 8 clearly demonstrated that the TR2 could repress the E2-induced ER transactivation in various cell lines.

[0066] Next we determined if the TR2 could repress the ER endogenous target gene expression. PR was chosen because it is well studied as an E2-ER target gene in T47D cells. Misrahi, et al., *Biochem. Biophys. Res. Commun.* 143, 740-748 (1987). After 24 h transient transfection of the pCMV-TR2 or parent vector pCMV, the T47D cells were treated with or without 10 nM E2 and cultured for another 24 h. Cells were then used for RNA isolation (Northern blot analysis) and lysate protein extraction (Western blot analysis). For RNA analysis, total RNAs were isolated, and Northern blots were performed using [^{32}P] labeled PR cDNA probes. The membrane was autoradiographed. For Protein Western blot analysis, cells lysates were prepared and PR proteins were visualized by radioimmunoblot. The membrane was autoradiographed to PhosphorImager and was quantitated by ImageQuant software. As shown in Fig. 9A, the PR mRNA expression was induced about 3 fold after E2 treatment. Addition of the TR2 clearly repressed the expression of endogenous PR mRNA in the presence of 10 nM E2. The Western blot analysis also clearly demonstrated that TR2 could repress the endogenous PR expression in T47D cells (Fig. 9B). Similar results were obtained when we replaced the T47D cells with another breast cancer cell line, MCF7. These *in vivo* TR2-mediated suppressive effects

strongly support the above reporter assays and demonstrate that TR2 can function as a repressor to repress ER target genes in breast cancer cells.

TR2 Prevents ER from Binding to its Target DNA.

[0067] The EMSA using [^{32}P]-labeled ERE as probe was applied to further dissect the mechanism of how the TR2 represses ER-mediated transactivation. 0.1 ng [^{32}P] end-labeled ERE oligomers (4 x 10^8 dpm/μg) were incubated with *in vitro* translated TR2 or ER proteins in EMSA binding buffer and analyzed on a 5% acryamide gel containing *2.5%* glycerol. 1 μl of Anti-ERα monoclonal antibody (C314) was used for supershifting. A 100-fold excess unlabeled ERE oligomer was used as a competitor. The specific ER-ERE band could be supershifted by adding ERα monoclonal antibody C-314. Addition of 100-fold unlabeled ERE oligonucleotides effectively eliminated this specific band. Interestingly, the intensity of this ER-ERE supershift decreased upon adding increasing amount of the TR2 in the absence or presence of 1μM E2. Together, these results suggest that the TR2 might be able to repress ER target genes by preventing the ER from binding to its target DNA. As there is no TR2-ERE specific band and no extra shifted band formed upon the addition of the TR2 to the ER-ERE complex, our data may also rule out the possibility of the formation of a transcriptional inactivated TR2-ER-ERE complex.

[0068] This conclusion was further confirmed by using the chimera receptor, TR2-androgen receptor (AR)ARp-TR2, which was generated by swapping TR2 proximal box (P-box) with that found in the AR. PC-3 cells were transiently co-transfected 2 μg of DR1-HBV-LUC reporter plasmid with 1 μg of pCMV, pCMV-TR2 or pSG5-TR2-ARp-TR2 in 35mm dishes. Cells were treated with 10^{-8} M E2 after 24 h transfection. Luciferase activity was then analyzed following manufacturer's instructions (Promega). Cells also were transfected with a β-galactosidase internal control reporter to correct for transfection efficiency. We found that the TR4 target DR1-HBV-LUC activity could be induced only by wild-type TR2 but not TR2-ARp-TR2 (Fig. 10A). We then tested the effect of TR2-ARp-TR2 on the ERE-CAT activity. H1299 and PC-3 cells were transiently co-transfected with 1 μg of ERE-CAT reporter plasmid with 1 μg of ER expression plasmid together with increasing amounts ofTR2-ARp-TR2 expression vector. CAT activity in the presence of 10^{-8} M E2. Cells also were transfected with a β-galactosidase internal control reporter to correct for transfection efficiency. We found that the ERE-CAT activity could be also repressed by TR2-ARp-TR2 in a dose-dependent manner in H1299 and PC-3 cells (Fig. 10B). Together, these results support the GST pull-down assay and EMSA data

by suggesting the possible mechanism of TR2 suppressive effect is through the interaction of ER with TR2-LBD, rather than through the TR2-DBD to compete out the binding between ER and ERE.

[0069] Other Interactions.

[0070] Using similar procedures, the interactions between the TR2 receptor and the AR receptor were studied. It was found that the TR2 receptor can suppress transactivation by the AR receptor. Similarly, studies were undertaken to evaluate the interaction between the the RXR receptor and the AR, and it was found that the RXR receptor can also suppress transactivation of the AR receptor. Hence, several mechanism exist to permit suppression of the AR receptor through co-suppression by an allied nuclear receptor.

CLAIM OR CLAIMS

I/WE CLAIM:

1. A method for screening a compound for use in treatment of androgen related diseases comprising the steps of

testing the compound to determine the effect of the compound on nuclear receptor mediated transcriptional activity, the activity being mediated by a nuclear receptor selected from the group consisting of the TR2 orphan receptor, the TR4 orphan receptor, and the RXR receptor, and

observing the effect of such compound on the level of androgen receptor initiated transcription in the test.

2. A method for screening a compound for use in treatment of estrogen related diseases comprising the steps of

testing the compound to determine the effect of the compound on nuclear receptor mediated transcriptional activity, the activity being mediated by a nuclear receptor selected from the group consisting of the TR2 orphan receptor, the TR4 orphan receptor, and the RXR receptor, and

observing the effect of such compound on the level of estrogen receptor initiated transcription in the test.

3. A method for modulating the sensitivity of a cell to a sex hormone comprising the step of stimulating in the cell the abundance of a nuclear receptor selected from the groups consisting of the TR2 orphan receptor, the TR4 orphan receptor and the RXR receptor.

4. A method for modulating androgen receptor-mediated transactivation activity in a cell, comprising the step of:

treating the cell with a compound that can modulate TR2 orphan receptor level or TR4 orphan receptor level in the cell.

5. A method for down regulating androgen receptor-mediated transactivation activity in a cell, comprising the step of:

introducing TR2 orphan receptor ligand binding domain or TR4 orphan receptor ligand binding domain into the cell.

6. A method for modulating estrogen receptor-mediated transactivation activity in a cell, comprising the step of:

treating the cell with a compound that can change TR2 orphan receptor level or the TR4 orphan receptor level in the cell.

7. The method of claim 6 wherein the agent is TR2 receptor.

8. The method of claim 6 wherein the compound is the TR2 orphan receptor ligand binding domain.

9. A method for down regulating TR2 orphan receptor-mediated transactivation activity in a cell, comprising the step of:

introducing estrogen receptor ligand binding domain into the cell.

10. A method for screening a compound for treating androgen receptor-related diseases, comprising the step of:

exposing cells to the compound; and

determining the effect of the compound on TR2 or TR4 orphan receptor signaling pathway in the cells.

11. The method of claim 10, wherein the effect on TR4 orphan receptor signaling pathway is measured by TR4 orphan receptor level.

12. The method of claim 10, wherein the effect on TR4 orphan receptor signaling pathway is measured by TR4 orphan receptor-mediated transactivation activity.

13. A method for screening a compound for treating estrogen receptor-related diseases, comprising the step of:

exposing cells to the compound; and

determining the effect of the compound on TR2 orphan receptor signaling pathway in those cells.

14. The method of claim 13 wherein the effect on TR2 orphan receptor signaling pathway is measured by TR2 orphan receptor level.

15. The method of claim 13, wherein the effect on TR2 orphan receptor signaling pathway is measured by TR2 orphan receptor-mediated transactivation activity.

ABSTRACT OF THE DISCLOSURE

Androgen receptor and TR4 orphan receptor have been found to heterodimerize with each other. Estrogen receptor and TR2 orphan receptor have been found to heterodimerize with each other. The heterodimer formation represses the transactivation activity of both receptors of each heterodimer. The finding is useful for new treatment options and new therapeutic agent screening methods for nuclear receptor related diseases and clinical conditions.

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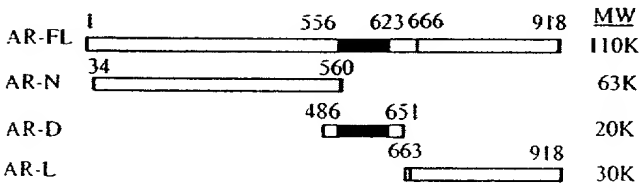
AR-FL
1
556
623
666
918
MW
110K
AR-N
34
560
63K
AR-D
486
651
20K
AR-L
663
918
30K

FIG 1

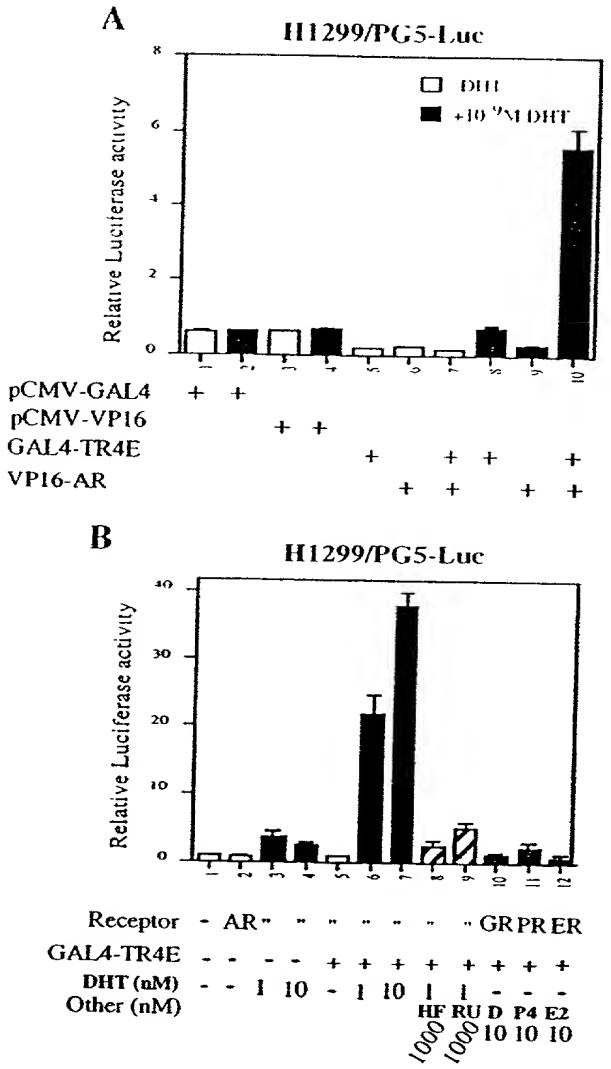
A
H1299/PG5-Luc
DHT
+10 nM DHT
Relative Luciferase activity
pCMV-GAL4
pCMV-VP16
GAL4-TR4E
VP16-AR
B
H1299/PG5-Luc
Relative Luciferase activity
Receptor
GAL4-TR4E
DHT (nM)
Other (nM)
AR
GR PR ER
HF RU D P4 E2
1000 1000 10 10 10

FIG 2

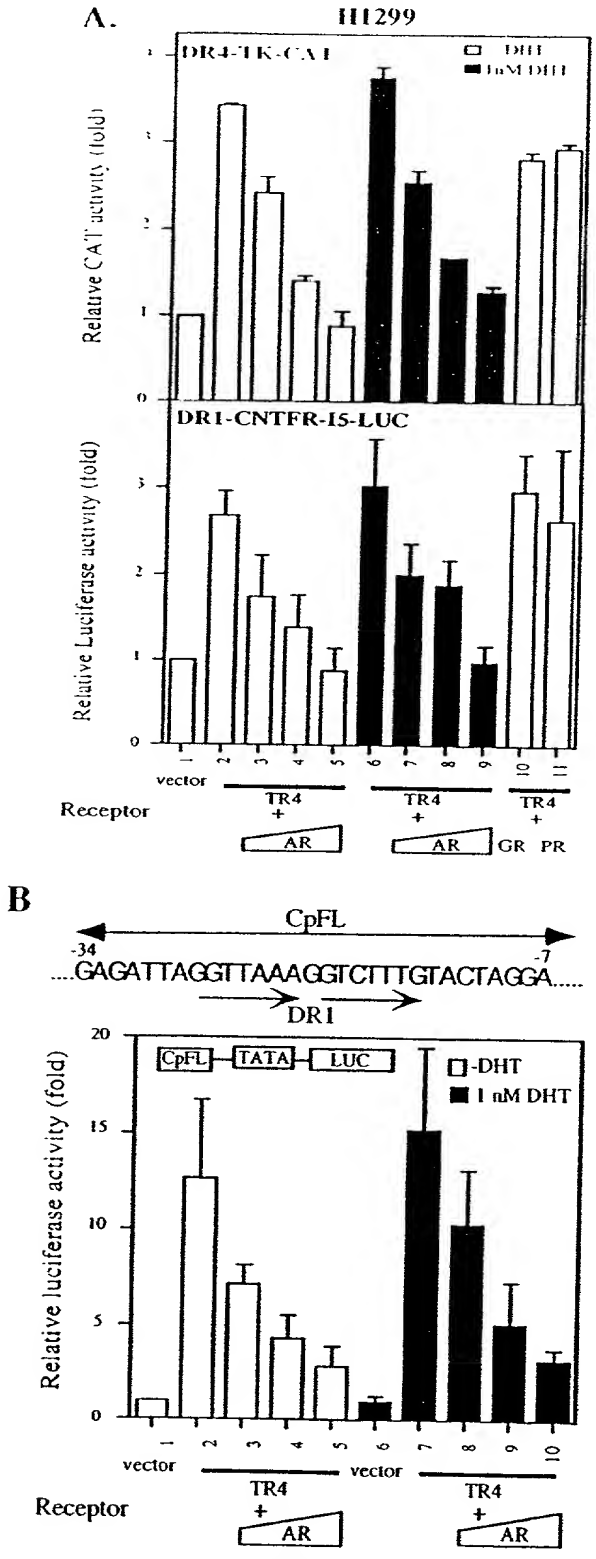
A.
H1299
DR4-TK-CAT
DHT
1nM DHT
Relative CAT activity (fold)
DR1-CNTFR-15-LUC
Relative Luciferase activity (fold)
vector
TR4
+
AR
GR
PR
Receptor
B
CpFL
-34
-7
....GAGATTAGGTTAAAGGTCTTTGTACTAGGA.....
DR1
CpFL
TATA
LUC
-DHT
1 nM DHT
Relative luciferase activity (fold)
vector
TR4
+
AR
Receptor

FIG 3

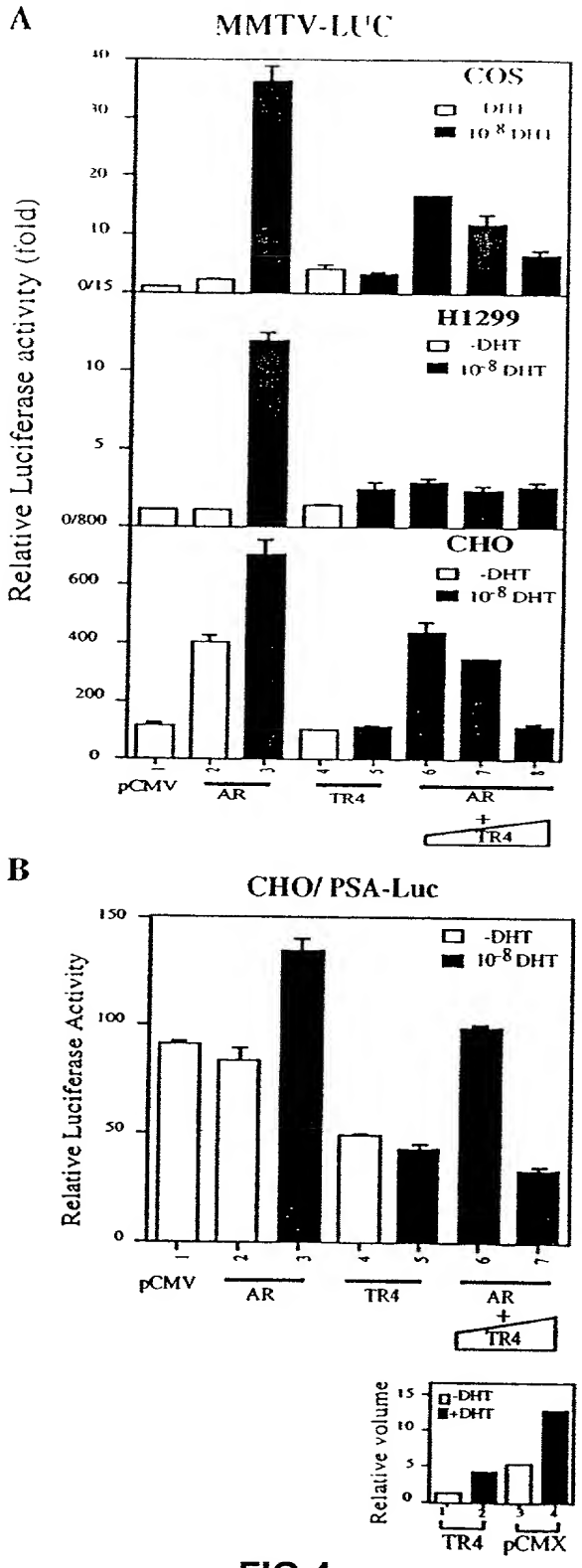
A
MMTV-LUC
COS
-DHT
10-8 DHT
H1299
-DHT
10-8 DHT
CHO
-DHT
10-8 DHT
Relative Luciferase activity (fold)
pCMV
AR
TR4
AR
+
TR4
B
CHO/ PSA-Luc
-DHT
10-8 DHT
Relative Luciferase Activity
pCMV
AR
TR4
AR
+
TR4
Relative volume
-DHT
+DHT
TR4
pCMX

FIG 4

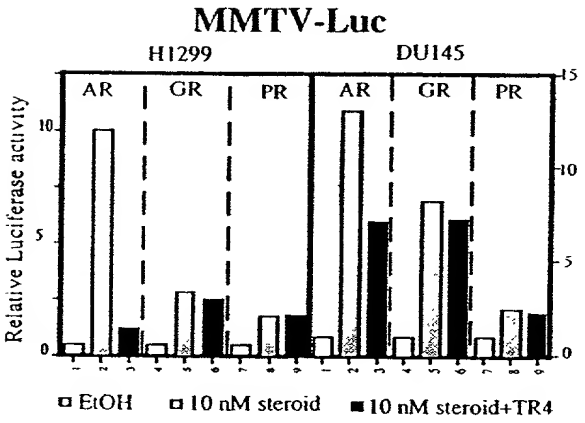
MMTV-Luc
H1299
DU145
AR
GR
PR
AR
GR
PR
Relative Luciferase activity
0
5
10
15
EtOH
10 nM steroid
10 nM steroid+TR4

FIG 5

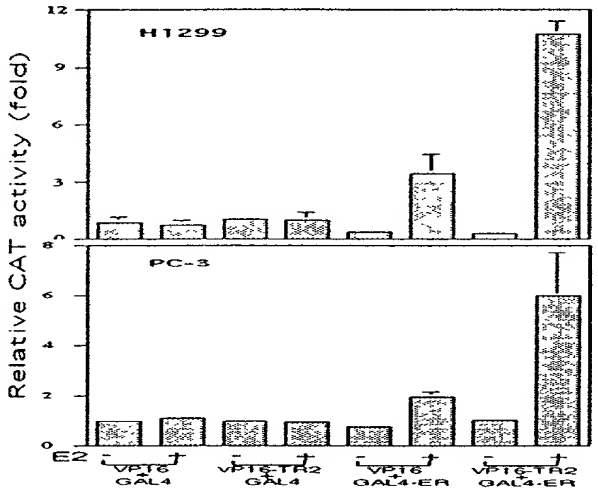
H1299
PC-3
Relative CAT activity (fold)
E2
VP16
GAL4
VP16-TR2
GAL4
VP16
GAL4-ER
VP16-TR2
GAL4-ER

FIG 6

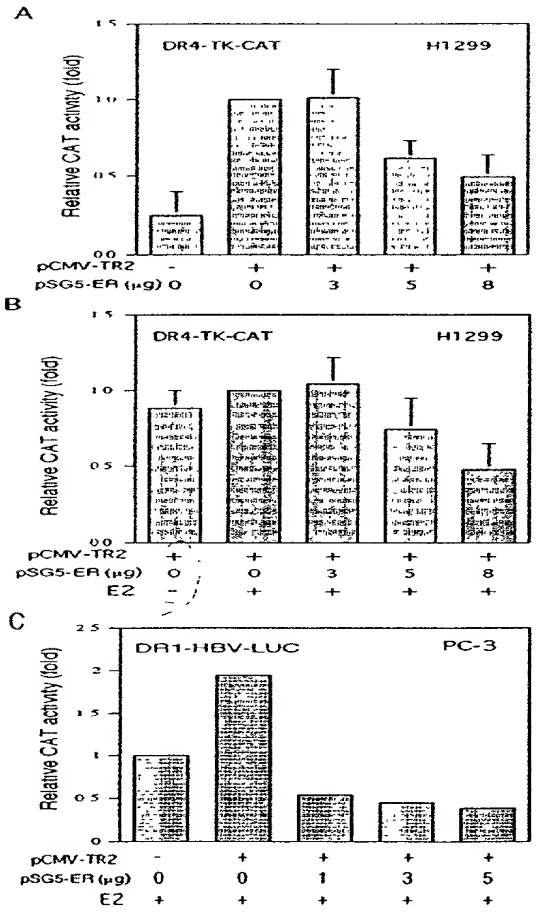
A
DR4-TK-CAT
H1299
Relative CAT activity (fold)
pCMV-TR2 - + + + +
pSG5-ER (μg) 0 0 3 5 8
B
DR4-TK-CAT
H1299
Relative CAT activity (fold)
pCMV-TR2 + + + + +
pSG5-ER (μg) 0 0 3 5 8
E2 - + + + +
C
DR1-HBV-LUC
PC-3
Relative CAT activity (fold)
pCMV-TR2 - + + + +
pSG5-ER (μg) 0 0 1 3 5
E2 + + + + +

FIG 7

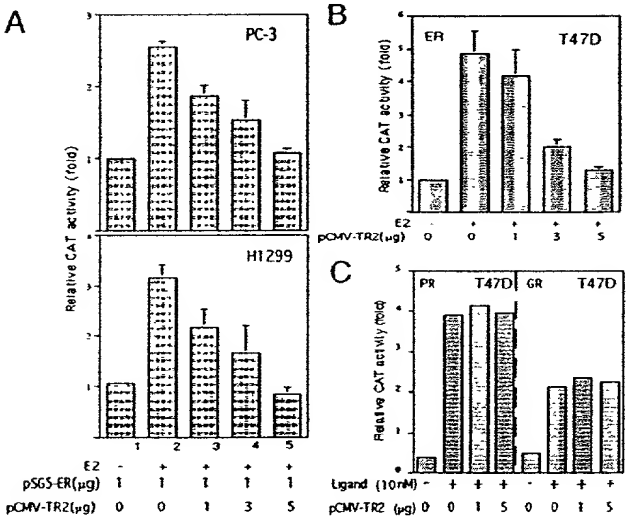
A
PC-3
H1299
Relative CAT activity (fold)
E2
pSG5-ER(μg)
pCMV-TR2(μg)
B
ER
T47D
Relative CAT activity (fold)
E2
pCMV-TR2(μg)
C
PR
T47D
GR
T47D
Relative CAT activity (fold)
Ligand (10nM)
pCMV-TR2 (μg)

FIG 8

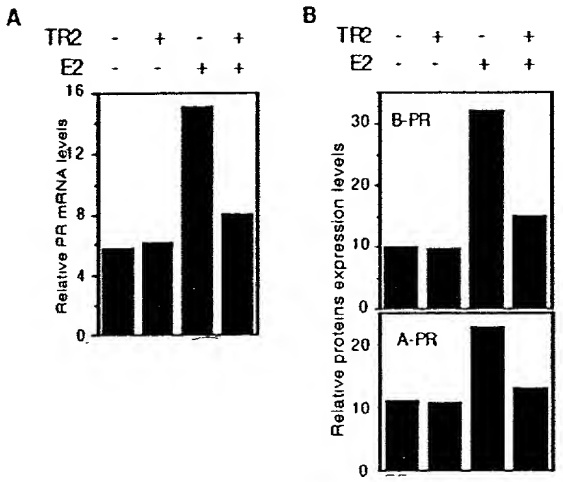
A
TR2 - + - +
E2 - - + +
Relative PR mRNA levels
16
12
8
4
0
B
TR2 - + - +
E2 - - + +
B-PR
A-PR
Relative proteins expression levels
30
20
10
0
20
10
0

FIG 9

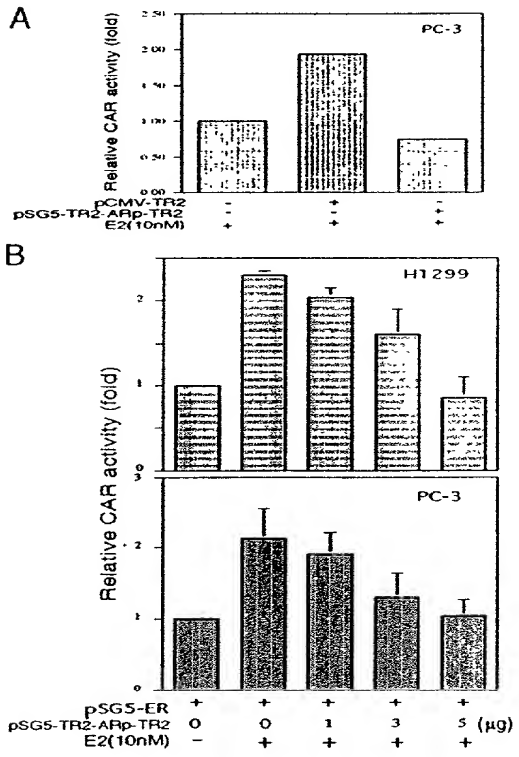
A
PC-3
Relative CAR activity (fold)
pCMV-TR2
pSG5-TR2-ARp-TR2
E2(10nM)
B
H1299
PC-3
Relative CAR activity (fold)
pSG5-ER
pSG5-TR2-ARp-TR2
E2(10nM)
0
1
3
5 (μg)

FIG 10

EXPRESS MAIL LABEL NO. EK941917725US

PTO/SB/01 (6-95)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Please type a plus sign (+) inside this box [+]

0010/PTO Rev. 6/95 — U.S. Department of Commerce Patent and Trademark Office

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing OR [ ] Declaration Submitted after Initial Filing

Attorney Docket Number	920920.90045
First Named Inventor	Chawnshang Chang
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MUTUAL SUPPRESSION BETWEEN SEX HORMONE RECEPTORS AND OTHER NUCLEAR RECEPTORS

(Title of the Invention)

the specification of which

[X] is attached hereto

OR

[ ] was filed on (MM/DD/YYYY) _____ as United States Application Number or PCT International Application Number _____ and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/165,300	November 12, 1999

[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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Please type a plus sign (+) inside this box [+]

DECLARATION

I hereby claim benefit under Title 35, United States Code §120 of any United States application(s), or §365(C) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT international application in the manner provided in the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

[ ] Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and all continuation and divisional applications based thereon, and to transact all business in the Patent and Trademark Office connected therewith:

[ ] Firm Name _____ Customer Number or label _____

OR

[X] List attorney(s) and/or agent(s) name and registration number below

Name	Registration Number	Name	Registration Number
Herbert W. Mylius	24,578	Bruce Neel	37,406
Barry E. Sammons	25,608	Michael A. Jaskolski	37,551
Charles Jirauch	26,186	Richard T. Roche	38,599
Nicholas J. Seay	27,386	Alexander Ching	41,669
George E. Haas	27,642	Terri S. Flynn	41,756
Michael J. McGovern	28,326	John T. Pienkos	42,997
Carl R. Schwartz	29,437	Daniel G. Radler	43,028
Keith M. Baxter	31,233	Gregory M. Smith	43,136
John D. Franzini	31,356	Steven J. Wietrzny	44,402
Janine Novat	32,593	Paul D. Amrozowicz	45,264
Jean C. Baker	35,433	David M. Kettner	45,598
David G. Ryser	36,407	Adam J. Forman	46,707
Bennett J. Berson	37,094		

[ ] Additional attorney(s) and/or agents named on a supplemental priority sheet attached hereto

Please direct all correspondence to [ ] Customer Number or label _____ OR [X] Fill in correspondence address below

Name	Nicholas J. Seay				
Address	Quarles & Brady LLP				
Address	P O Box 2113				
City	Madison	**State**	WI	**Zip**	53701-2113
Country	USA	**Telephone**	(608)251-5000	**Fax**	(608)251-9166

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of Sole or First Inventor: [ ] A petition has been filed for this unsigned inventor

Given	Chawnshang	Middle		Family	Chang	Suffix	

Inventor's Signature		Date	

Residence:	Pittsford	State	NY	Country	US	Citizenship	

Post Office	
Post Office	

City	Pittsford	State	WI	Zip		Country	US	Applicant Authority	

[ ] Additional inventors are being named on supplemental sheet(s) attached hereto